Available online at www.sciencedirect.com

SCIENCE DIRECT®

Developmental Biology 293 (2006) 565 – 580

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Genomes & Developmental Control

The canonical Notch/RBP-J signaling pathway controls the balance of cell lineages in mammary epithelium during pregnancy

Krista D. Buono [a], Gertraud W. Robinson [a], Cyril Martin [a], Shaolin Shi [b,1], Pamela Stanley [b], Kenji Tanigaki [c], Tasuku Honjo [c], Lothar Hennighausen [a,*]

[a] *Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA*

[b] *Department of Cell Biology, Albert Einstein College of Medicine, New York, NY 10461, USA*

[c] *Department of Medical Chemistry, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan*

Received for publication 5 January 2006; revised 16 February 2006; accepted 18 February 2006

Abstract

Mammary alveoli are composed of luminal (secretory) and basal (myoepithelial) cells, which are descendants of a common stem cell. This study addressed the role of RBP-J-dependent Notch signaling in the formation, maintenance and cellular composition of alveoli during pregnancy. For this purpose, the genes encoding RBP-J, the shared transcriptional mediator of Notch receptors, and Pofut1, a fucosyltransferase required for the activity of Notch receptors, were deleted in mammary progenitor cells in the mouse using Cre-mediated recombination. Loss of RBP-J and Pofut1 led to an accumulation of basal cell clusters characterized by the presence of cytokeratins (K5) and K14 and smooth muscle actin (SMA) during pregnancy. Hormonal stimulation of mutant tissue induced the expression of the basal cell transcription factor p63 in luminal cells and excessive proliferation of basal cells. A transient enrichment of K6-positive luminal cells was observed upon hormonal treatment suggesting a temporary arrest at an immature stage prior to transdifferentiation and expansion as basal cells. Despite the extensive proliferation of RBP-J-null basal cells during pregnancy, hormonal withdrawal during involution resulted in complete remodeling and the restoration of normal tissue architecture. We propose that the Notch-RBP-J pathway regulates alveolar development during pregnancy by maintaining luminal cell fate and preventing uncontrolled basal cell proliferation.

Keywords: Notch; Myoepithelium; Basal cells; Pofut1; RBP-J; Mammary epithelium; Cytokeratins

Introduction

The Notch family of cell surface receptors controls the specification of a wide variety of cell types (Schweisguth, 2004; Tanigaki et al., 2003). Evidence for a link between Notch signaling and mammary tumorigenesis came from observations that integration of the mouse mammary tumor virus (MMTV) into an intron of the *Notch4 (int3)* gene leads to the formation of mammary tumors (Gallahan and Callahan, 1987). In this case, transcription initiated in the MMTV-LTR leads to hybrid transcripts that encode the constitutively active Notch4 Intra-Cellular-Domain (ICD) (Gallahan and Callahan, 1997). Moreover, expression of the Notch4 ICD under control of mammary-specific regulatory elements in transgenic mice confirmed that activation of Notch signaling leads to the establishment of mammary tumors (Gallahan et al., 1996; Jhappan et al., 1992; Kordon et al., 1995; Raafat et al., 2004). Expression of the Notch4 ICD under control of the *WAP* gene promoter resulted in an initial block of epithelial cell proliferation and differentiation during pregnancy (Gallahan et al., 1996) suggesting a genuine participation of Notch4 in normal development. However, mice from which the *Notch4* gene had been inactivated by homologous recombination were able to lactate (Krebs et al., 2000), suggesting that either Notch4 is not required for normal mammary development or that other Notch family members fill the void in its absence. Expression of Notch1 ICD under the control of MMTV-LTR induces tumors during lactation that regress after involution (Kiaris et al., 2004), identifying a potential role for another Notch receptor in mammary gland development.

* Corresponding author. Fax: +1 301 480 7312.
E-mail address: lotharh@mail.nih.gov (L. Hennighausen).

[1] Present address: Dept. Medicine, Division of Nephrology, Mt. Sinai School of Medicine, New York, NY 10461, USA.

0012-1606/$ - see front matter
doi:10.1016/j.ydbio.2006.02.043

The functional unit in the mammary gland during lactation is the alveolus, which produces and secretes milk. An alveolus consists of two distinct cell types, the luminal or secretory cells and the basal or myoepithelial cells, both of which appear to originate from one common alveolar progenitor cell (Kordon and Smith, 1998; Smith and Boulanger, 2003). Development of mammary tissue occurs in distinct stages and is controlled by systemic hormones and local growth factors (Hennighausen and Robinson, 2001, 2005). The mammary anlage is established at around day 11.5 of fetal development and a rudimentary ductal tree develops prior to birth. Development of the extended ductal tree is driven by estrogen and progesterone during puberty. Formation of the alveolar compartment is controlled by the prolactin receptor (Ormandy et al., 1997), the kinase Jak2 (Shillingford et al., 2002), and the transcription factor Stat5 (Cui et al., 2004; Miyoshi et al., 2001).

The signals that determine the cell fate switch from the common progenitor to the basal and luminal compartments have not been identified. Since aberrant Notch signaling can disrupt the differentiation state of mammary epithelium, we postulated a role for this developmental cue in the lineage commitment of mammary progenitors. As it is not feasible to use mouse genetics to simultaneously inactivate all four Notch receptors, we addressed the role of Notch signaling using two approaches that address distinct aspects of the Notch signaling pathway. First, RBP-J, the common downstream partner of all Notch ICDs, was inactivated. Second, Pofut1, a fucosyltransferase essential for the activity of Notch receptors (Okajima and Irvine, 2002; Sasamura et al., 2003; Shi and Stanley, 2003), was inactivated. By inactivating Notch signaling using two distinct mechanisms, potential Notch-independent effects of RBP-J (Beres et al., 2006; MacKenzie et al., 2004) would be revealed. Since loss of the *Rbpsuh* (Oka et al., 1995) and *Pofut1* (Shi and Stanley, 2003) genes results in fetal death, we deleted the two genes specifically in mammary progenitor cells of the mouse using Cre-mediated recombination (Wagner et al., 1997, 2001). Using antibodies against specific basal and luminal proteins, it was possible to monitor on a single cell level molecular consequences of the absence of RBP-J and Pofut1. This approach also permitted an analysis of how basal and luminal cells respond to pregnancy hormones in the presence and absence of RBP-J and Pofut1.

Materials and methods

Antibodies

Antigen	Antibody species	Provider	Product no.	Antigen retrieval (pH)	Dilution
Cre	Rabbit	Novagen	69050-3	9.0	1:200
E-cadherin	Mouse	BD Transduction Laboratories	610181	6.0	1:100
ERα	Rabbit	Santa Cruz	sc-542	6.0	1:100
Keratin 14	Sheep	The Binding Site	Discontinued	9.0	1:100
Keratin 14	Rabbit	Panomics	E2624	9.0	1:200
Keratin 18	Mouse	RDI (Research Diagnostics Inc)	RDI-PRO61028	9.0	1:100
Keratin 5	Rabbit	Covance	PRB-160P	9.0	1:1000
Keratin 6	Rabbit	Covance	PRB-169P	9.0	1:200
Keratin 8	Sheep	The Binding Site	Discontinued	9.0	1:100
PCNA	Mouse	DAKO	M 0879	9.0	1:200
Progesterone	Rabbit	DAKO	A0098	6.0	1:100
p63	Mouse	NeoMarkers	MS-1081-P	6.0	1:200
SMA	Mouse	Sigma	A 2547	9.0	1:1000
Stat-5a	Rabbit	Santa Cruz	sc-1081	9.0	1:100 O/N 4°C
Alexa Fluor® 488	Goat	Molecular Probes, Inc.	A-11001		1:400
Alexa Fluor® 488	Goat	Molecular Probes, Inc.	A-11008		1:400
Alexa Fluor® 488	Donkey	Molecular Probes, Inc.	A-11015		1:400
Alexa Fluor® 594	Goat	Molecular Probes, Inc.	A-11005		1:400
Alexa Fluor® 594	Goat	Molecular Probes, Inc.	A-11012		1:400
Alexa Fluor® 594	Donkey	Molecular Probes, Inc.	A-11016		1:400

Mouse breeding and genotyping

Mice which carry floxed *Rbpsuh* (Han et al., 2002) and *Pofut1* (Shi et al., 2005; Shi and Stanley, 2003) alleles were in a mixed 129/C57BL/6 background. Mice were generated that carried either two *Rbpsuh* or two *Pofut1* floxed alleles and the *MMTV-Cre* transgene (line A) (Wagner et al., 2001). The mice were treated according to the animal protocols approved by the Animal Care and Use Committee at NIH.

PCR analysis was used for determining the genotype of these mice. RBP-J wild-type intron was detected using primers: wt1: 5′-gTTcTTAAccTgTTggTcg-gAAcc-3′ and wt2: 5′-gcTTgAggcTTgATgTTcTgTATTgc-3′ (Han et al., 2002). For the detection of the floxed allele, primers from the neomycin cassette were used. neo1: 5′-AgAggcTATTcggcTATgAcTg-3′ and neo2: 5′-TTcgTccAgAT-cATccTgATc-3′ *Pofut1* wild-type and floxed alleles were detected by PCR using primers 644: 5′-AcccAcAggcTgTgcAgTcTTTg-3′ and 645: 5′-gggTcAccTT-cATgTAcAAgTgAgTg-3′ (95°C, 5 min; 35 cycles: 95°C, 1 min, 62°C, 1 min, 72°C, 1 min; 72°C, 7 min).

Primers for the Cre transgene are as follows: 5′-ggTTcTgATcTgAgcTcT-gAgTg-3′ binding in the MMTV long terminal repeat and 5′-cATcAcTcgTTg-cATcgAccgg-3′ binding in the Cre sequence.

Histology and immunohistochemistry

Mammary glands were fixed in 10% neutral buffered formalin overnight at 4°C. After fixation, tissues were placed in 70% ethanol, dehydrated and paraffin-embedded. For histology, sections were stained with hematoxylin and eosin (H&E). For immunostaining, paraffin sections (5 μm) were cleared in xylene and rehydrated through an alcohol series. Digital Decloaking Chamber (Biocare Medical; Walnut Creek, CA) was utilized for antigen retrieval. Sections were immersed in BORG*DECLOAKER* or Reveal 1× (heat-induced epitope-retrieval solution, 9.5 pH or 6.0 pH, respectively). SP1 (Set-Point1) was 125°C for 5 min and SP2 (Set-Point2) was at 90°C for 10 s. After carefully rinsing slides with running tap water, the sections were placed in PBS containing 0.05% (v/v) Tween-20 (PBST). After blocking in PBST with 3% goat serum for 1 h, primary antibodies were applied (see above for dilution). Sections were incubated with PBST containing primary antibody for 1 h at 37°C and washed in PBS. Stat5a and cleaved Caspase 3 antibodies were incubated overnight at 4°C with PBST containing 3% goat serum. Fluorescence-conjugated secondary antibodies (1:400) were applied to sections for 30 min in the dark at room temperature, washed in PBS, and mounted with VectaShield with DAPI (Vector Laboratories; Burlingame, CA). Sections were viewed under an epifluorescence equipped Olympus BX51 microscope with filters for DAPI, FITC, TRITC, and FITC/TRITC. Images were captured with a Q Imaging Retiga Exi digital camera (Image Systems, Inc.; Columbia, MD) and Image-Pro Plus 5.1 program. Brightness and contrast were adjusted using Adobe Photoshop software.

Transplantation

The transplantation technique has been described (DeOme et al., 1959). Donor mice were 3 to 4 weeks of age while recipients were 3-week-old female athymic nude (nu/nu) mice from which the endogenous mammary epithelium had been cleared by surgically removing the area between nipple and the lymph node. All control donors were *Rbpsuh*$^{fl/fl}$ or *Pofut1*$^{fl/fl}$ littermates. In addition, mammary anlagen from fetuses were used for transplants as described (Robinson et al., 2001). After 8 weeks, transplanted fat pads were removed for analysis at the virgin stage. Transplanted mice were also mated, plug checked, and then harvested at defined pregnancy time points, after parturition and in involution. For serial transplantation, small pieces of tissues were taken from the first and second generation transplants and transplanted into cleared fat pads.

Hormone treatment

Eight weeks after receiving transplants, hosts were treated for 2 days with daily subcutaneous injections of 1 μg β-estradiol (E) (Sigma-Aldrich E-8515) and 1 mg progesterone (P) (Sigma-Aldrich P-0130) in 100 μl of sesame oil. Transplants were harvested 24 h later and were processed for histology.

Results

Aberrant epithelial development upon inactivation of the Rbpsuh gene in mammary progenitor cells

The *Rbpsuh* gene was inactivated using Cre-loxP-mediated recombination. *Rbpsuh*$^{fl/fl}$ mice (Han et al., 2002) were bred with a well-defined line of transgenic mice expressing Cre recombinase under control of the MMTV-LTR (Wagner et al., 1997, 2001). This *MMTV-Cre* (MC) transgene is active already in mammary progenitor cells in the newborn and genes flanked by loxP sites are deleted in both basal (myoepithelial) and luminal (secretory) cells of ducts and alveoli (Supplementary Fig. 1). In addition to mammary epithelium, this *MMTV-Cre* transgene is also expressed in hematopoietic cells, skin, and hair follicles (Wagner et al., 1997, 2001). *Rbpsuh*$^{fl/fl;MC}$ mice appeared overtly normal at birth but could be identified within a few days by a scaly appearance of the skin. These mice failed to thrive, displayed skin lesions similar to those described (Yamamoto et al., 2003), and died within a few weeks after birth (data not shown). It was therefore necessary to transplant mammary epithelium from mutant mice into wild-type hosts. Development was analyzed during and after pregnancy and in involution. Eight weeks after transplantation, the ductal tree was overtly normal (Supplementary Fig. 2), demonstrating that RBP-J is not required for the establishment of ductal basal and luminal cells.

Development of mammary alveoli during pregnancy is achieved through the proliferation and differentiation of luminal and basal cells. Using histological analyses, no differences were evident between *Rbpsuh*$^{fl/fl;MC}$ and control mammary tissue at day 4 of pregnancy (Figs. 1A and E). At day 7 of pregnancy, the overall extent of ductal branching in mutant tissue appeared normal (data not shown) but on the histological level profound defects in the epithelial architecture were observed (Fig. 1F). The immature alveoli in wild-type tissue consisted of round cells with large nuclei positioned radially around the central lumen, which is small at this stage (Fig. 1B). In *Rbpsuh*$^{fl/fl;MC}$ tissue, epithelial cells were arranged irregularly with frequent gaps between them (Fig. 1F). In addition, many of the lumina contained cellular debris. On day 11 of pregnancy, whole mounts of wild-type tissue exhibited numerous alveoli while *Rbpsuh*$^{fl/fl;MC}$ epithelia appeared much sparser (Supplementary Fig. 2). On day 13 of pregnancy, the lumina in wild-type tissue were enlarged and contained lipid droplets and proteinaceous material, clear signs of a secretory differentiation (Fig. 1C). In the majority of *Rbpsuh*$^{fl/fl;MC}$ epithelia, clusters of disorganized cells with irregularly shaped nuclei were seen. Lipid droplets and lumina were rare (Fig. 1G). At term, wild-type tissue displayed alveoli that were large and distended. The cells lining the lumina were differentiated and secreted milk (Fig. 1D). In contrast, in *Rbpsuh*$^{fl/fl;MC}$ tissue, the number and size of disorganized cell clusters had further increased compared to day 13 of pregnancy. The majority of the cells had dark irregularly shaped nuclei that surrounded small groups of cells with larger round nuclei (Fig. 1H).

Paucity of luminal cells and excess of cells with a basal character

While basal cells are characterized by the presence of cytokeratins 5 (K5) and 14 (K14) and smooth muscle actin (SMA), luminal cells feature the presence of the transcription factor Stat5, the estrogen receptor alpha (ERα), and cytokeratins 8 (K8) and 18 (K18). To further characterize the identity of the cells developing in *Rbpsuh*$^{fl/fl;MC}$ epithelium, antibodies against proteins expressed in luminal and basal cells were used. Moreover, the expression of the *MMTV-Cre* transgene in *Rbpsuh*$^{fl/fl;MC}$ mice was monitored by immunostaining (Fig. 2). During pregnancy, the MMTV-LTR is active in luminal but not basal cells, and correspondingly Cre was detected in ductal and alveolar luminal cells at day 4 of pregnancy (Fig. 2E). At this stage, alveoli and ducts were surrounded by a single layer of cells positive for SMA. These structures were similar in control and mutant tissues (Figs. 2A and E). At day 7 of pregnancy, MMTV-Cre expression was observed in cells positioned in the center of clusters that were reminiscent of the luminal compartment of ducts and immature alveoli. These cells were surrounded by multilayered SMA expressing cells (Fig. 2F). MMTV-Cre expression at day 13 of pregnancy was confined to clusters of cells contained in a broad sheath of irregularly arranged SMA-

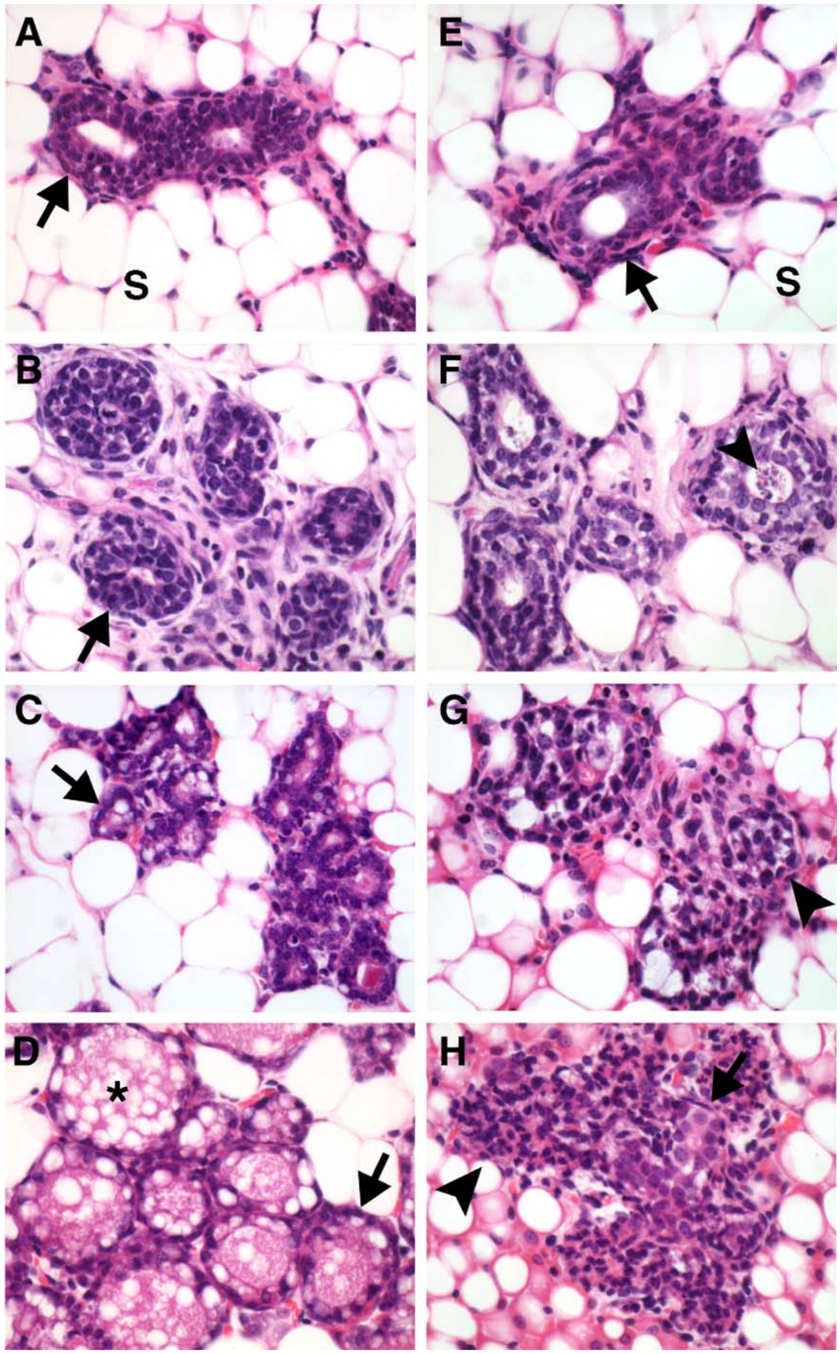

Fig. 1. Alveolar morphology of *Rbpsuh*$^{fl/fl;MC}$ mammary tissue. H&E staining of sections from control (A–D) and *Rbpsuh*$^{fl/fl;MC}$ mammary tissues (E–H). On day 4 of pregnancy, no significant differences were apparent between control (A) and *Rbpsuh*$^{fl/fl;MC}$ (E) tissues. Multilayered luminal cells discernible by their round shape were surrounded by elongated myoepithelial cells (arrows). On day 7 of pregnancy, alveoli in control tissue (B) consisted of compact clusters of luminal cells around a small central lumen and an outer layer of myoepithelial cells (arrow). Alveoli in *Rbpsuh*$^{fl/fl;MC}$ tissue (F) contained irregularly arranged luminal cells with frequent gaps between them. Cellular debris was found in the lumina (arrowhead). On day 13 of pregnancy, lipid droplets were seen in the luminal cells of control tissue (arrow) (C). *Rbpsuh*$^{fl/fl;MC}$ tissue contained alveolar-like structures that lacked a lumen and consisted of cells with irregularly shaped nuclei (arrowhead) (G). At parturition, alveoli in control tissue (D) had a distended lumen (asterisk) that was filled with milk and milk fat globules, the luminal cells displayed signs of secretory differentiation, and basal (myoepithelial) cells (arrow) were stretched at the periphery of the alveoli. The epithelial compartment in *Rbpsuh*$^{fl/fl;MC}$ tissue (H) consisted of densely packed cells with irregular dark nuclei (arrowhead) with a few islands of round cells whose nuclei were less compact (arrow). S—stroma.

positive cells that did not express Cre (Fig. 2G). At the same stage, alveoli in control tissue were small and round and contained a monolayer of SMA-positive cells (Fig. 2C). Immediately after parturition, very few MMTV-Cre expressing cells were detected (Fig. 2H), further demonstrating the dearth of genuine luminal cells.

The abundance of SMA-positive cells in later stages of pregnancy prompted us to investigate whether these cells

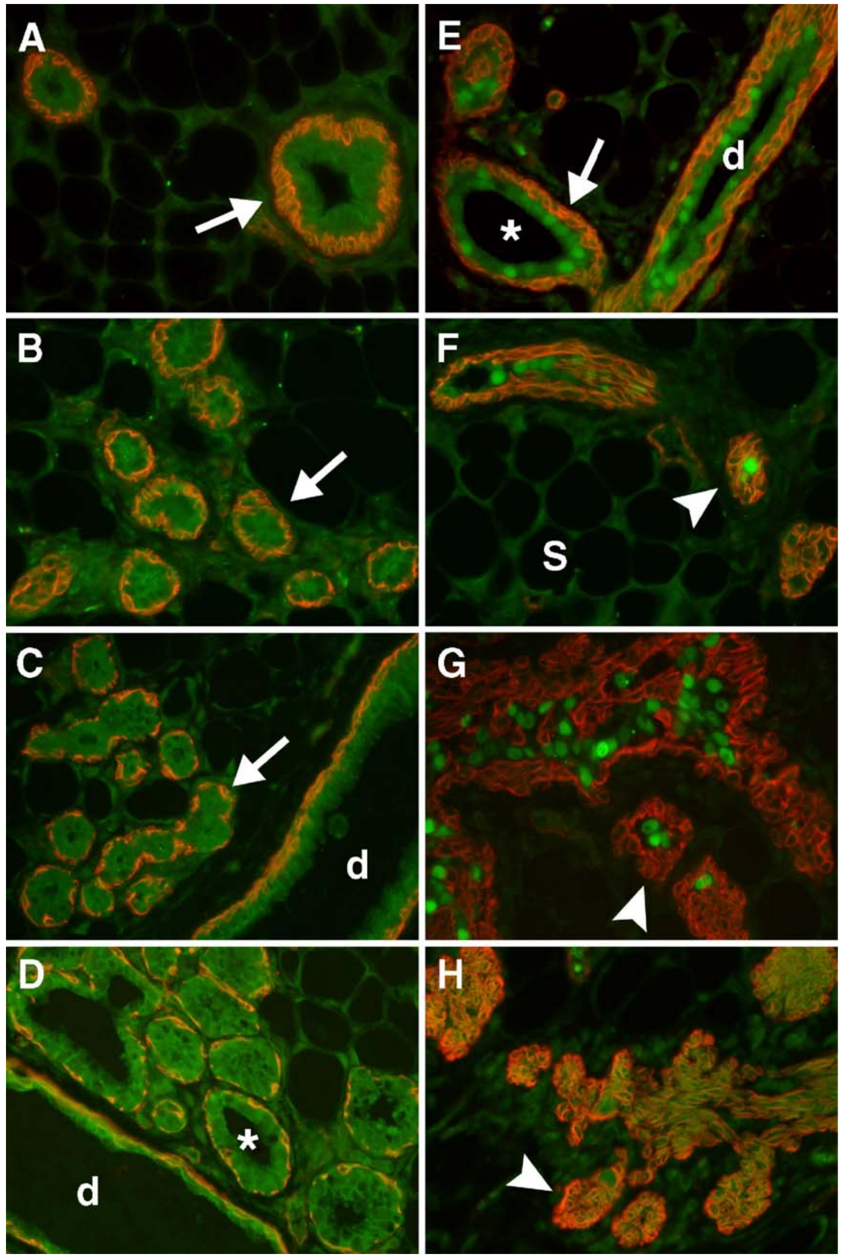

Fig. 2. Expression of MMTV-Cre and SMA in *Rbpsuh*$^{fl/fl;MC}$ tissue during pregnancy. Cre recombinase expression (green fluorescence) was seen in nuclei of luminal cells in *Rbpsuh*$^{fl/fl;MC}$ tissue at days 4 (E), 7 (F), and 13 (G) of pregnancy. At parturition (H), Cre was still found in some sporadic cells. SMA staining of basal cells (red fluorescence, arrow) demonstrated an increase in basal cells as pregnancy progressed. On day 13 and at term, the tissue consisted of densely packed basal cells (arrowheads). Tissues from non-transgenic animals of the same stages are shown in panels A to D as controls. The asterisk indicates open lumina. S—stroma; d—ducts.

displayed characteristics of *bona fide* basal cells. Sections from control and *Rbpsuh*$^{fl/fl;MC}$ tissues were stained with anti-K14 and anti-K18 antibodies. The multilayered cells in *Rbpsuh*$^{fl/fl;MC}$ tissue were positive for K14 (Fig. 3), thus confirming their basal character. While basal and luminal cells from control tissue displayed K14 and K18, respectively, some centrally located cells from mutant mammary tissue coexpressed K14 and K18 at all stages examined during pregnancy (Fig. 3). The appearance of K14/K18 positive cells was first evident at day 4 of pregnancy (Fig. 3E) and suggested that luminal cells can acquire basal characteristics in the absence of RBP-J.

Accumulation of cytokeratin 6 (K6) in luminal cells in the absence of RBP-J

The presence of cytokeratin 6 (K6) has been linked to the proliferation of luminal mammary alveolar epithelium at early stages of pregnancy (Smith et al., 1990) and with mammary stem and/or progenitor cells (Li et al., 2003b). In agreement with earlier studies (Smith et al., 1990), K6 was detected only very rarely in a few alveolar luminal cells at day 4 of pregnancy (Fig. 4A), but not at later stages or during lactation (Figs. 4B–D). In contrast, in the

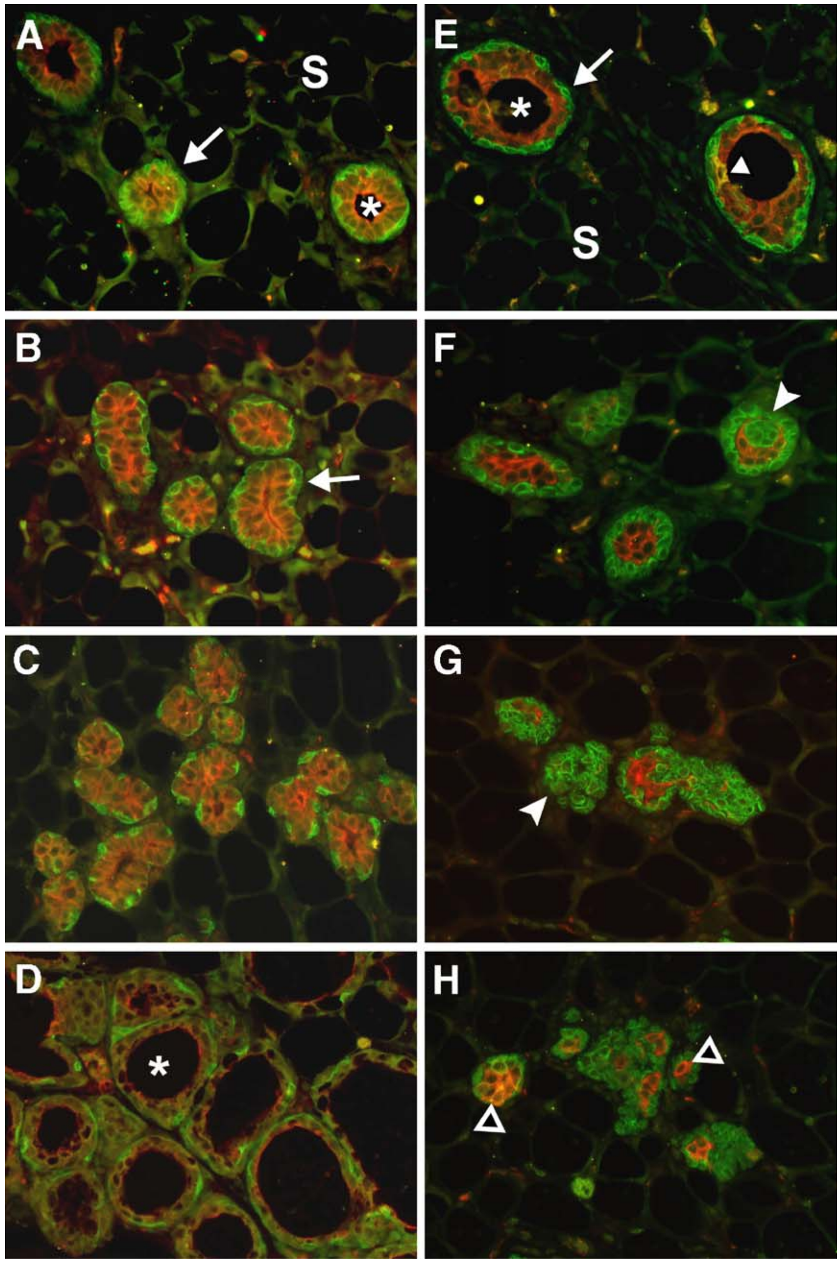

Fig. 3. Expansion of basal cells in *Rbpsuh*$^{fl/fl;MC}$ tissue during pregnancy. Expression of K14 (green fluorescence) was restricted to basal cells in control tissue at days 4 (A), 7 (B), and 13 (C) and at parturition (D). Luminal cells expressed K8 (red fluorescence). In *Rbpsuh*$^{fl/fl;MC}$ tissue at day 4 of pregnancy (E), K14 staining was preferentially found in basal cells. In some areas, coexpression of K18 and K14 was seen in luminal cells (arrowhead). At day 7 of pregnancy (F), luminal cells were surrounded by multiple layers of K14 expressing cells (arrowhead). An increase of K14 positive cells at the expense of K18 positive cells was seen at day 13 of pregnancy (G). At term, the majority of cells expressed K14 (H). Cells expressing K18 (arrowhead) were sporadically interspersed in dense clusters of K14 positive cells (open arrowhead). The asterisk indicates open lumina. S—stroma.

absence of RBP-J, approximately 50% of alveolar and ductal units contained K6-positive cells (Fig. 4E). In some alveoli, between 30–50% of cells in the luminal compartment were K6-positive. While K6-positive cells were still observed in one third of alveolar structures at day 13 of pregnancy (Fig. 4G), their presence was only sporadic after parturition (Fig. 4H). At early stages of pregnancy, K6-positive cells did not appear to express basal markers, such as K14, but at later stages overlap of K6 and K14 was observed. This further supports our observations that luminal cells undergo transdifferentiation in the absence of RBP-J.

The presence of the transcription factor Stat5 is a hallmark of functional mammary alveolar luminal cells (Liu et al., 1996). Indeed, proliferation and differentiation of these cells are fully dependent on the presence of the Stat5 genes (Cui et al., 2004; Miyoshi et al., 2001). While in control tissue strong nuclear Stat5a staining was seen in all luminal cells at day 13 of

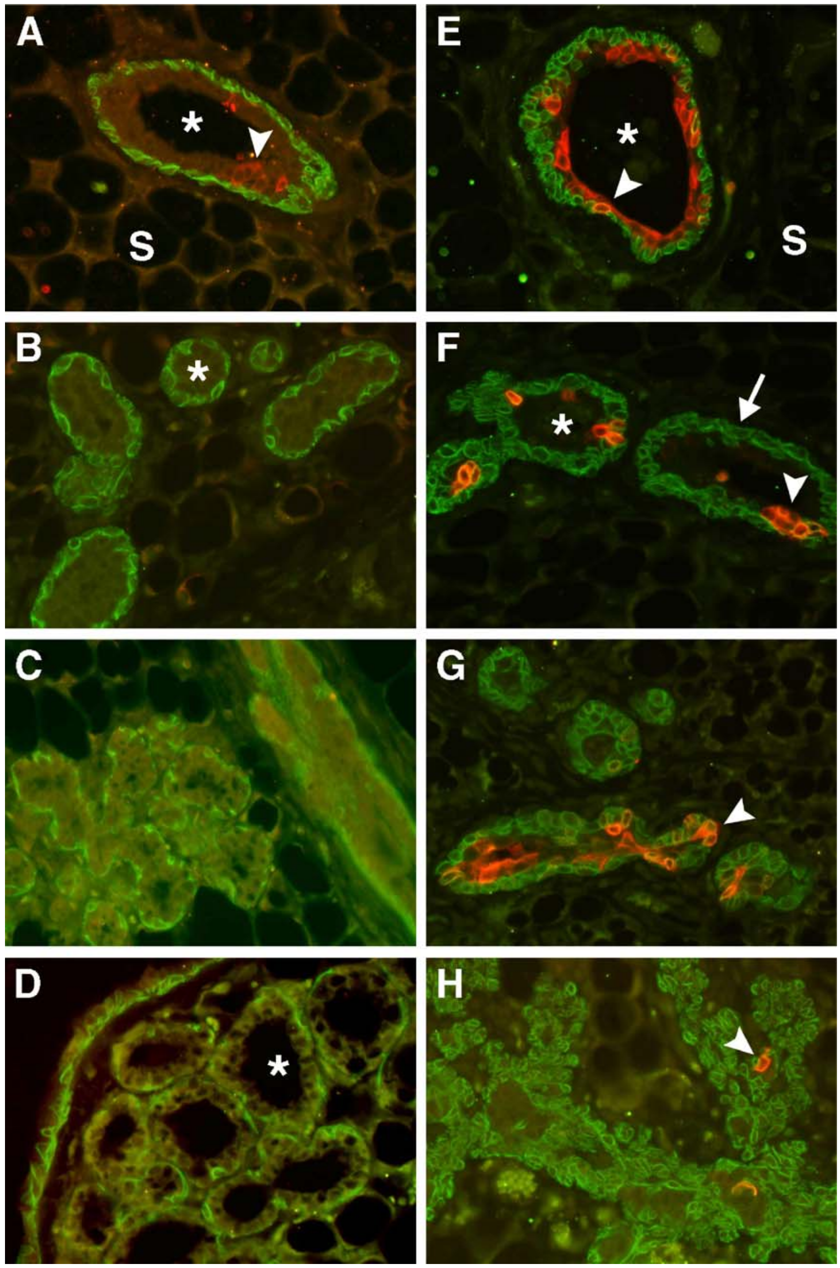

Fig. 4. Transiently expanded expression of K6 in *Rbpsuh*$^{fl/fl;MC}$ epithelia. In control tissue, K6 (red, arrowhead) was found at day 4 of pregnancy in a small number of cells that stained negative for the basal K14 (green, arrow) (A). The proportion of cells expressing K6 (red) is much greater in *Rbpsuh*$^{fl/fl;MC}$ tissue at the same stage (E). No K6 was detected at later stages in control tissue (B–D). In *Rbpsuh fl/fl-MC* epithelia, extensive K6 was detected also at pregnancy day 13 (G) but little expression was observed at term (H). Asterisk indicates the lumen. S—stroma.

pregnancy, only a few cells located in small clusters in the center of the epithelial units in *Rbpsuh*$^{fl/fl;MC}$ tissue were positive for Stat5a (Supplementary Fig. 3).

Luminal cells acquire p63

The transcription factor p63, an ancient member of the p53 family, is required for the establishment of epithelial structures including the mammary anlage (Yang et al., 1999). Its presence has been associated with basal or progenitor cells of skin (for review see McKeon (2004)). In control tissue, p63 was observed in basal but not luminal cells at all stages of pregnancy and at parturition (Figs. 5A–D). While the presence of p63 coincided with that of K5 (Figs. 5A and C), K8-positive cells did not stain for p63 (Figs. 5B and D). Similarly, in *Rbpsuh*$^{fl/fl;MC}$ tissue at day 4 of pregnancy, p63 colocalized with K5-positive basal cells and not with K8-positive luminal cells (Figs. 5E and F, respectively). However, mutant tissues contained an excess of clustered p63-positive cells. At parturition, p63 was abundant not only in the basal cell layer but throughout the multilayered K5-positive cells (Fig. 5G). Importantly, a subset of p63-positive cells were K5-negative (Fig. 5G) and positive for K8 (Fig. 5H). This demonstrates that the absence of RBP-J leads to the expression of basal markers in luminal cells.

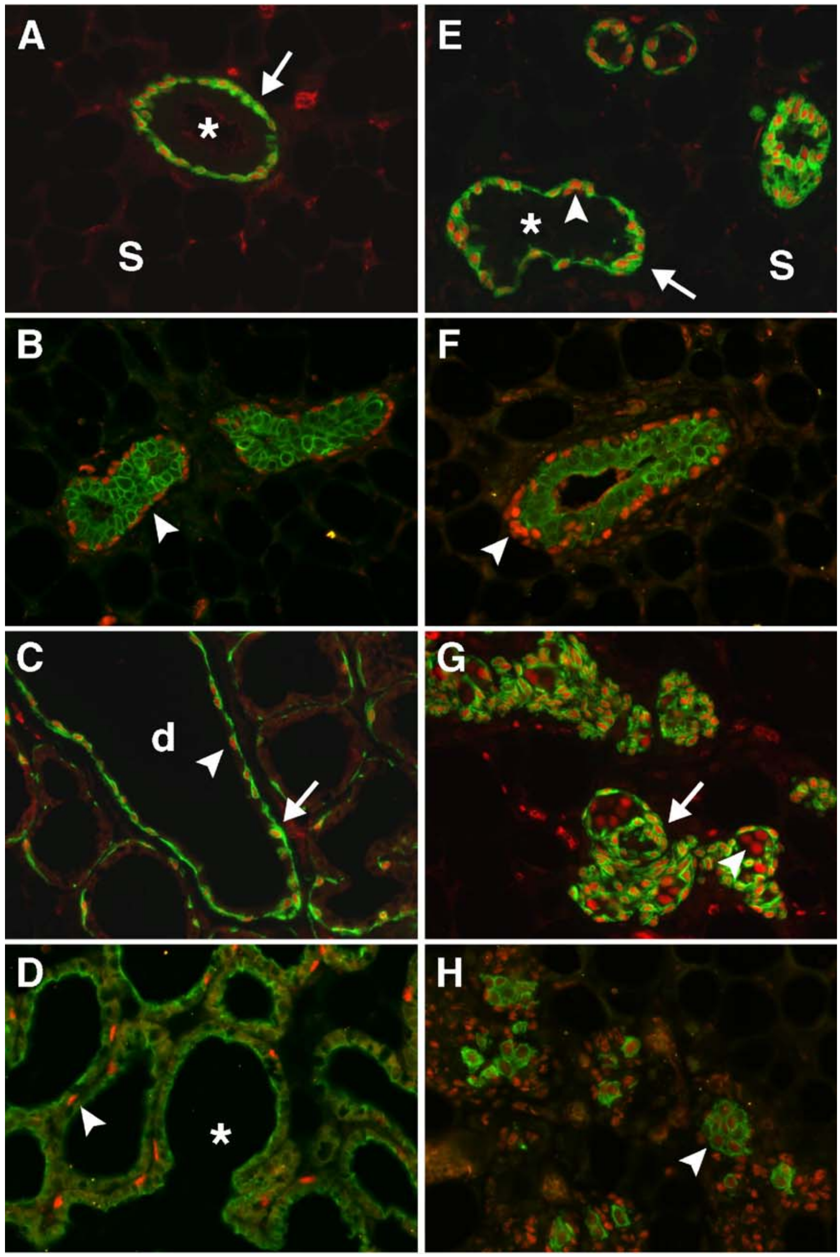

Fig. 5. Aberrant p63 expression in *Rbpsuh*$^{fl/fl;MC}$ epithelia. At day 4 of pregnancy, p63 (red fluorescence, arrowhead) was observed in cells that also expressed the basal marker K5 (green fluorescence, arrow) in both control (A) and *Rbpsuh*$^{fl/fl;MC}$ tissue (E). No p63 staining (red) was seen in K8 (green) positive cells at this stage (B and F). Note the irregular spacing of p63 positive cells at the periphery of *Rbpsuh*$^{fl/fl;MC}$ alveoli (F). At term, p63 (red) and K5 (green) (C) but not K8 (green) (D) were colocalized in control tissue. In *Rbpsuh*$^{fl/fl;MC}$ epithelia, p63 (red) was found in cells that expressed K5 (G) as well as in cells positive for K8 (green) (H). Arrowheads point to p63 expressing cells. The asterisk indicates open lumina. S—stroma.

Basal cells proliferate in the absence of RBP-J

In normal mammary tissue during pregnancy, proliferation is extensive in luminal cells but rarely seen in basal cells (Joshi et al., 1986; Sapino et al., 1990). Although the sporadic appearance of cells coexpressing basal and luminal markers indicates conversion of luminal cells into basal cells, this event alone cannot account for the dramatic accumulation of basal cells in the absence of RBP-J. Therefore, cell proliferation was investigated throughout pregnancy (Fig. 6). In control tissues, approximately 77% and 37% of the cells were PCNA-positive at pregnancy days 4 and 13, respectively. In *Rbpsuh*$^{fl/fl;MC}$ mutant tissues from the same stages, approximately 51% and 54% of cells were PCNA-positive. Little proliferation was observed at parturition in both control and mutant tissues. While proliferation in control tissue occurred almost exclusively in luminal cells, proliferation in mutant tissue was seen preferentially in basal cells (Fig. 6). The ratios of proliferating basal and luminal cells were determined in mutant and wild-type tissue at day 13 of pregnancy. While in control tissue 12-times more luminal cells were PCNA positive than basal cells, in mutant tissue 10-times more basal cells than luminal cells were PCNA

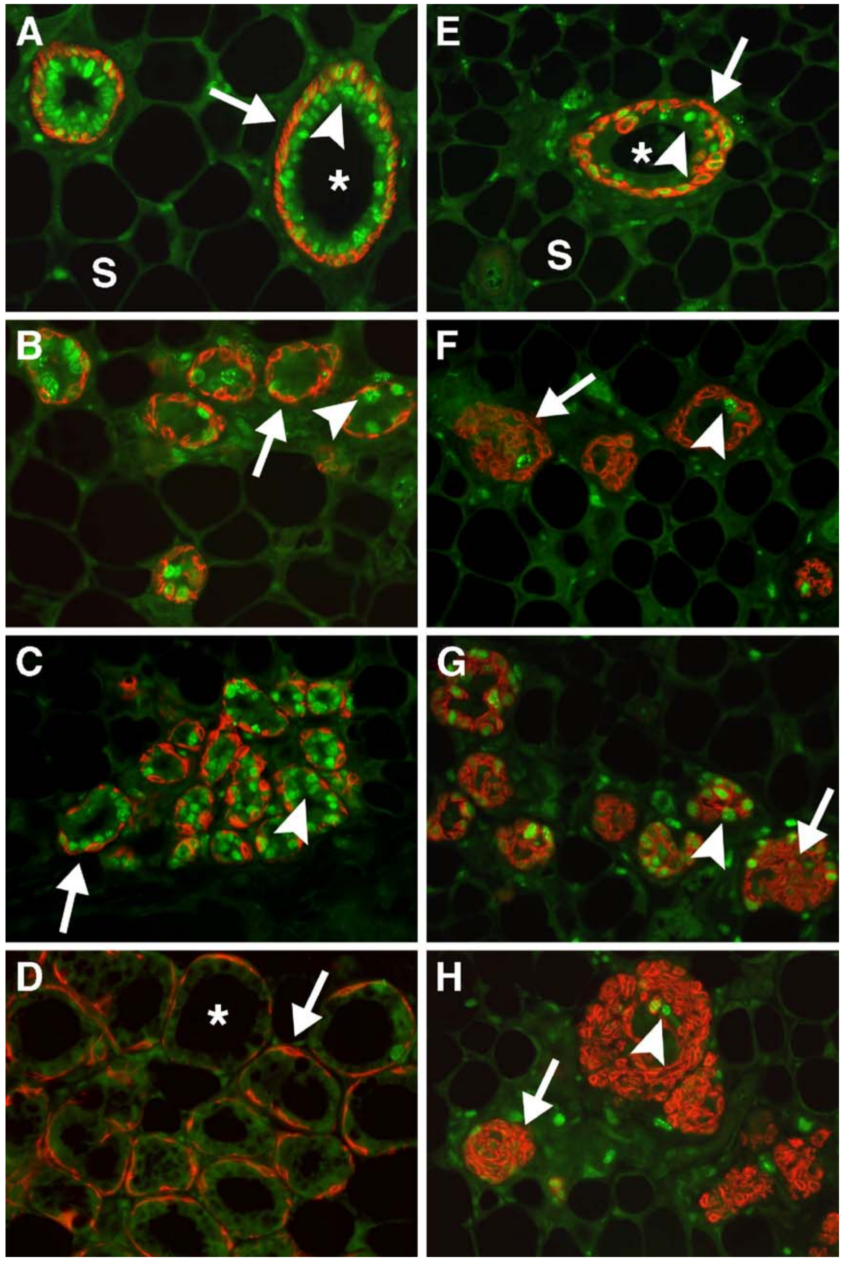

Fig. 6. Basal cell proliferation during pregnancy in *Rbpsuh*$^{fl/fl;MC}$ epithelia. At day 4 of pregnancy, PCNA staining (green fluorescence, arrowhead) of luminal cells was reduced in *Rbpsuh*$^{fl/fl;MC}$ tissue (E) compared to controls (A). K5 is stained in red (arrows). PCNA staining is reduced in both tissues at day 7 of pregnancy (B and F). At day 13 of pregnancy, many PCNA positive luminal cells are seen in control tissue (C). In *Rbpsuh*$^{fl/fl;MC}$ epithelium, PCNA staining is found in cells that express K5 (red fluorescence) (G). At term, PCNA staining is absent in control tissue (D) while sporadic PCNA positive cells are found in both epithelial compartments of *Rbpsuh*$^{fl/fl;MC}$ tissue (H). Note the wide rim of K5 expressing cells in *Rbpsuh*$^{fl/fl;MC}$ alveoli (F through H). The asterisk indicates the lumen. S—stroma.

positive. These results clearly demonstrate that pregnancy-mediated proliferation normally observed in luminal cells is directed to the basal cells in the absence of RBP-J.

RBP-J-null luminal cells retain the estrogen receptor alpha (ERα)

While the estrogen receptor alpha (ERα) and progesterone receptors (PR) are present in mammary epithelial cells that do not proliferate, no expression is detected in proliferating cells (Anderson and Clarke, 2004). In the mouse, less than 50% of luminal cells are positive for ERα (Shyamala et al., 2002). It is generally believed that cells expressing the ERα and PR induce a paracrine response on neighboring cells to undergo proliferation. Since alveolar luminal cells failed to proliferate in the absence of RBP-J, an underlying mechanism could be a deregulation of ERα expression. As expected, ERα was present in control tissue at day 4 of pregnancy in a subset of luminal cells (Fig. 7A) but much reduced at term (Figs. 7B and C). In contrast, ERα was detected in virtually all luminal cells in

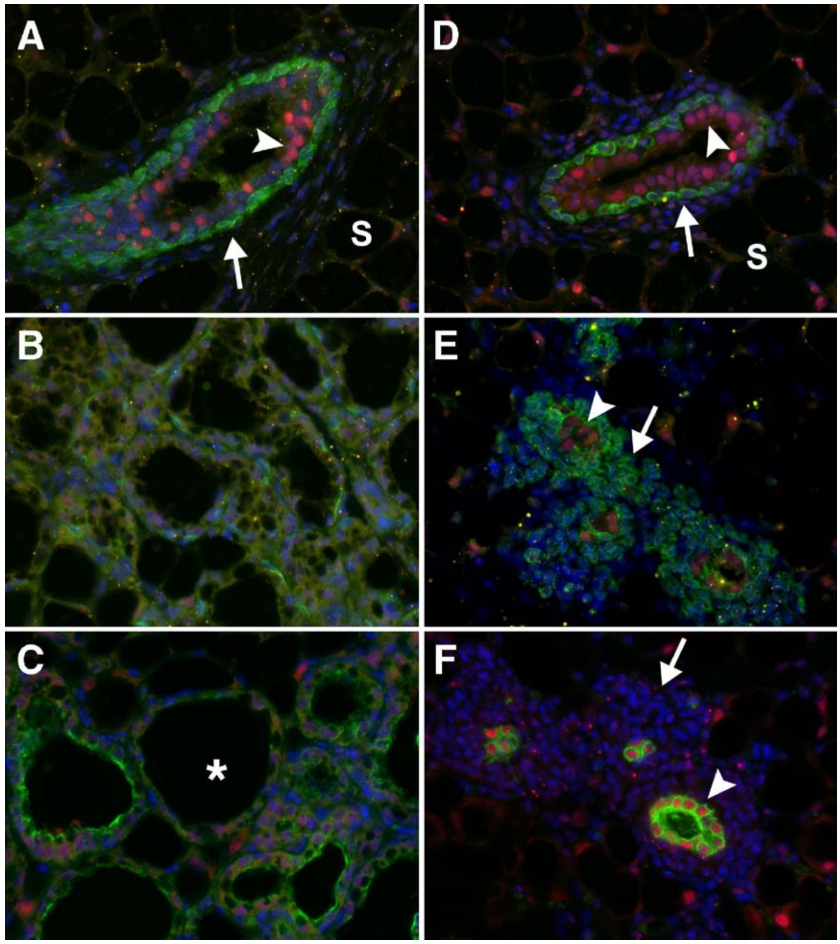

Fig. 7. Expression of estrogen receptor alpha in all luminal cells of *Rbpsuh*$^{fl/fl;MC}$ epithelia. At day 4 of pregnancy, mosaic expression of ERα (red fluorescence, arrowhead) was seen in some luminal cells of wild-type tissue (A) and in the majority of luminal cells in *Rbpsuh*$^{fl/fl;MC}$ tissue (D). K5 (arrow) staining is shown in green and nuclei are stained blue with DAPI. At parturition, ERα expression was absent in control tissue (B and C) but was still strong in the few remaining cells that express K18 in mutant tissue (F). No overlap of ERα staining and K5 was seen in *Rbpsuh*$^{fl/fl;MC}$ epithelia (E). K5 is stained in green in panels B and E and K18 is stained in green in panels C and F.

mutant tissue at day 4 of pregnancy (Fig. 7D). As pregnancy progressed, all K18-positive cells retained ERα and even at parturition the few remaining K18-positive cells expressed ERα (Fig. 7F). There was no overlap in expression between K5 and ERα (Fig. 7E). Similarly to ERα, PR expression was detected throughout the luminal compartment and retained even at term (data not shown). Increased and persistent expression of PR in luminal cells has been detected in other mice from which genes that control mammary development and cell proliferation have been deleted, including the *PrlR* and *Stat5* (Grimm et al., 2002).

Estrogen and progesterone induce p63, basal cell characteristics, and cell proliferation

The analysis of mammary tissue during pregnancy could not provide conclusive information on the nature of the hormones that induced p63 and K6 as well as basal cell characteristics and cell proliferation. To establish the nature of the primary response, an acute hormonal stimulation experiment was performed. Virgin mice carrying *Rbpsuh*$^{fl/fl;MC}$ mammary epithelial transplants were injected with estrogen and progesterone (EP) and mammary tissue was analyzed after 2 days (Fig. 8). The presence of Cre in mutant tissue demonstrated that EP had activated the *MMTV-Cre* transgene (Fig. 8E). While in control tissue, p63 was localized exclusively in basal cells, in mutant tissue p63 was also expressed in the majority of luminal cells (Fig. 8F). Similarly, K6 was present in the majority of luminal cells in mutant tissue (Fig. 8G) but rarely in cells from control tissue (Fig. 8C). In control tissue, extensive proliferation occurred only in luminal cells (Fig. 8D) while proliferation in mutant tissue was largely confined to cells expressing p63, both in the original basal cell layer and those in the luminal compartment that had acquired p63 (Fig. 8H).

Regenerative capacity of Rbpsuh$^{fl/fl;MC}$ *mammary epithelium*

A subset of aggressive and invasive breast cancers are characterized by the expression of basal cell markers (Nielsen et al., 2004). Since loss of RBP-J in mammary progenitor cells results in both the transdifferentiation of luminal cells and the excessive proliferation of basal cells, we analyzed two features of *Rbpsuh*$^{fl/fl;MC}$ mammary epithelium. First, we explored

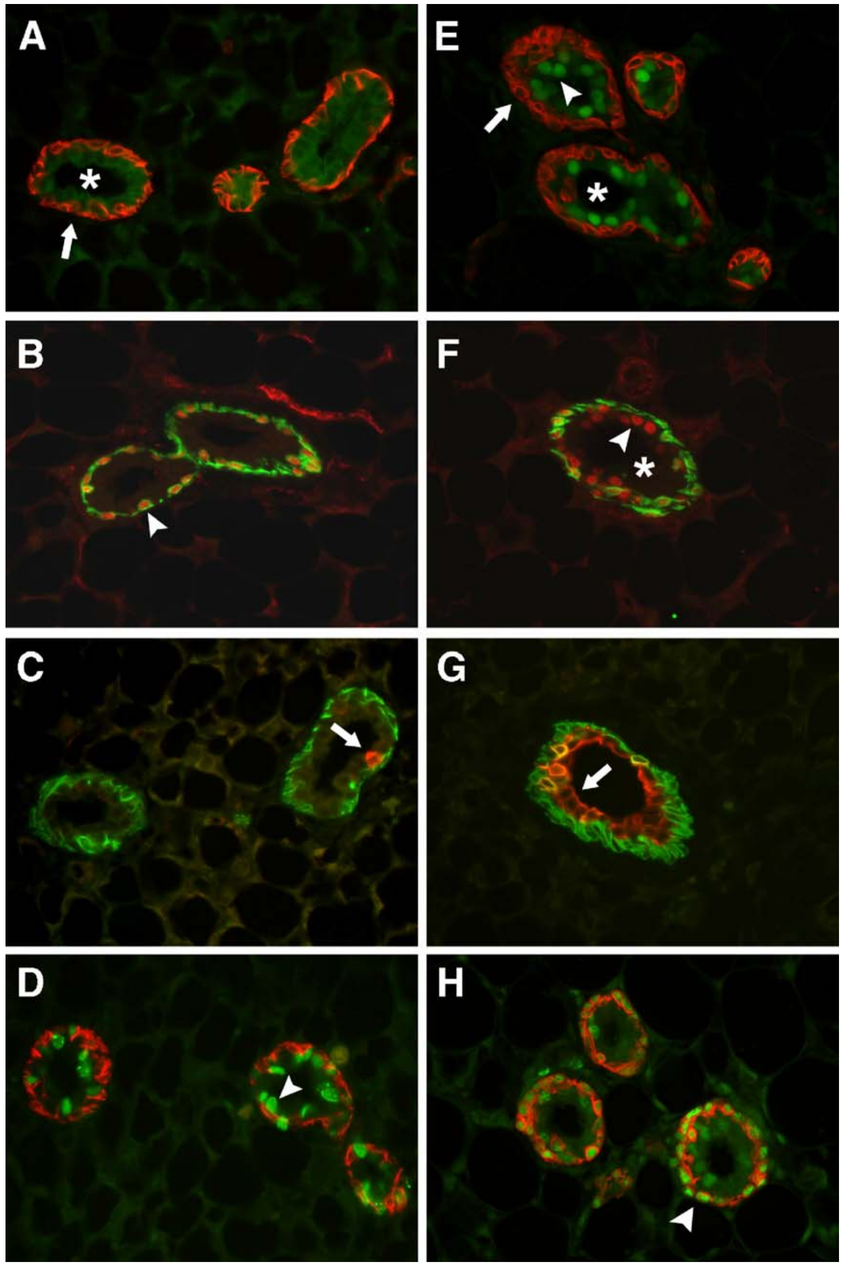

Fig. 8. Response of *Rbpsuh*$^{fl/fl;MC}$ epithelia to estrogen and progesterone. Estrogen and progesterone were injected into virgin mice harboring control and *Rbpsuh*$^{fl/fl;MC}$ mammary tissue, which were analyzed using immunostaining 2 days later. (A–D) Control tissue; (E–H) *Rbpsuh*$^{fl/fl;MC}$ tissue; (A and E) Cre (arrowhead) is stained in green and K5 in red; (B and F) p63 is stained in red and K5 in green; (C and G) K6 is stained in red and K14 in green; (D and H) PCNA is stained in red and K5 in red. Arrows in panels A and E point to basal cells. Arrows in panels C and G point to K6 expressing luminal cells.

whether mammary progenitor cells in the absence of RBP-J were able to continuously repopulate mammary tissue. Secondly, we analyzed whether *Rbpsuh*$^{fl/fl;MC}$ mammary epithelium had lost hormone-mediated growth control that is characteristic for normal mammary tissue.

In order to investigate the consequences of the absence of RBP-J in progenitor cells on long-term regeneration of mammary epithelium, *Rbpsuh*$^{fl/fl;MC}$ tissues were serially transplanted for three generations and evaluated at virgin, lactation, and involution stages. Ductal outgrowth and branching were not impaired in these transplants (Supplementary Fig. 4). The overall epithelial architecture was maintained and the size of alveolar buds did not increase compared to the original outgrowths. However, the proportion of basal cells in *Rbpsuh*$^{fl/fl;MC}$ virgin tissue after three successive transplants was increased compared to first generation tissue (Fig. 9B). A small number of luminal cells were present in the center of many of the alveolar like structures, suggesting that the mutant progenitor cells were still able to generate, at least transiently, cells with a luminal character. A small proportion of these cells expressed p63 but not the basal cell marker K5 while the expression of these proteins was colocalized and restricted to the basal layer in control tissue (Fig. 9A). Like the first generation transplant, *Rbpsuh*$^{fl/fl;MC}$ mammary epithelium

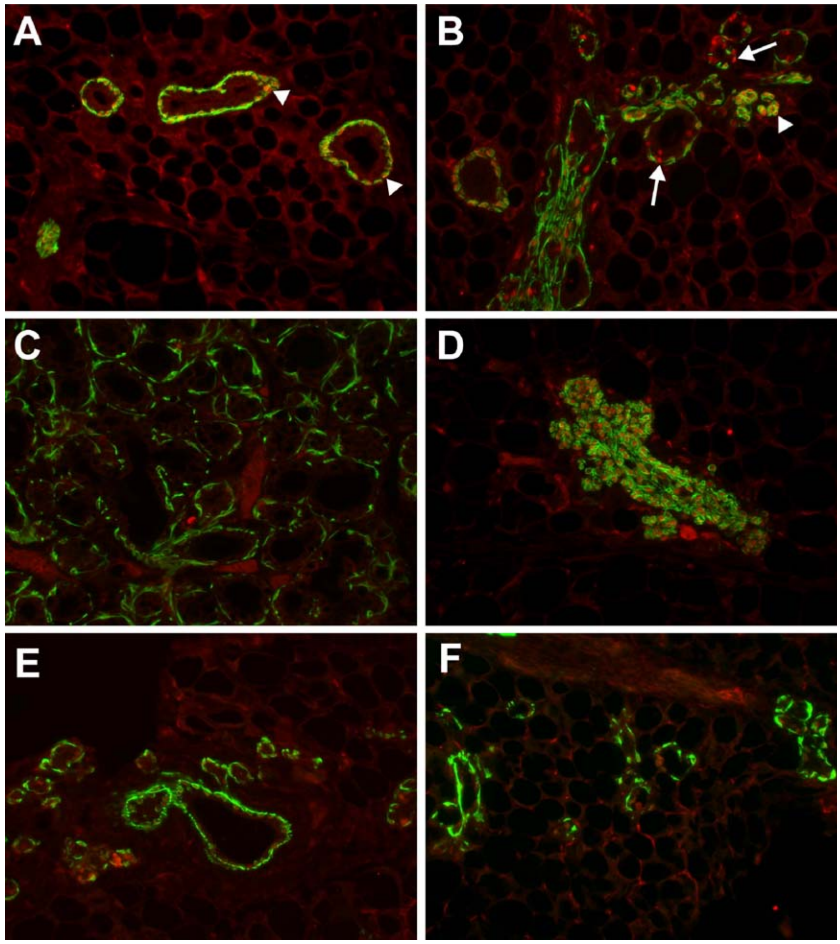

Fig. 9. Restoration of normal ductal architecture after involution. Third generation wild-type (A, C, and E) and *Rbpsuh*$^{fl/fl;MC}$ (B, D, and F) transplants were harvested at virgin stage (A and B), after one pregnancy (C and D), and 2 weeks after forced weaning of pups (E and F). In virgins, a single layer of myoepithelial cells which express p63 (red) and K5 is seen in wild-type tissue (arrowhead). Ducts of *Rbpsuh*$^{fl/fl;MC}$ tissue are heterogeneous and consist of regions with a single myoepithelial layer as well as multilayered areas. Expression of p63 is seen in cells that do not express K5 (arrow). At term, myoepithelial cells are stretched around the extended alveoli in wild-type tissue (C). *Rbpsuh*$^{fl/fl;MC}$ epithelium forms ducts that are composed almost exclusively of K5 (green) positive cells expressing p63 (red) (D). After involution, most of the alveolar cells have undergone apoptosis and a simple ductal system is restored in wild-type (E) and *Rbpsuh*$^{fl/fl;MC}$ epithelium (F).

transplanted for three times displayed a pregnancy-induced expansion of basal cells (Fig. 9D). Tissues that were subject to pregnancy and allowed to undergo involution for 2 weeks appeared similar to control tissues and the majority of p63 and K5 positive cells had disappeared (Figs. 9C and E). These results suggest that extended exposure to hormones in the estrous cycle increases the accumulation of myoepithelial cells. However, these cells do not display uncontrolled proliferation and are subject to normal cell death upon loss of lactogenic stimuli during involution.

Inactivation of the Pofut1 gene in mammary progenitor cells

Functional inactivation of the Notch receptors was achieved through the inactivation of the *Pofut1* gene. *O*-fucosyltransferase (Pofut1) is essential for the function of Notch receptors. *Pofut1*$^{fl/fl}$ mice (Shi and Stanley, 2003) were bred with the *MMTV-Cre* (MC) transgenic mice and *Pofut1*$^{fl/fl;MC}$ mice appeared overtly normal at birth but developed skin lesions reminiscent of those seen in the *Rbsuh*$^{fl/fl;MC}$ mice (Supplementary Fig. 5). Mammary tissue from these mice was transplanted into cleared recipients and development was investigated throughout pregnancy and parturition (Fig. 10). The developmental lesions observed were indistinguishable from those observed with *Rbpsuh*$^{fl/fl;MC}$ tissue. The disorganized alveolar morphology of *Pofut1*$^{fl/fl;MC}$ mammary tissue was similar to *Rbsuh*$^{fl/fl;MC}$ as demonstrated by histological analysis of mammary tissue at parturition (Fig. 10A). The epithelial compartment in *Pofut1*$^{fl/fl;MC}$ tissue consisted of densely packed cells with irregular dark nuclei. The abnormal expression patterns of the basal markers SMA (Fig. 10B), K14 (Fig. 10C), K5, and p63 (Fig. 10D) demonstrated that *Pofut1*$^{fl/fl;MC}$ mammary tissue had acquired a basal phenotype. These results demonstrate that the defects observed are due to the loss of the canonical Notch/RBP-J pathway.

Discussion

Mammary alveoli develop and undergo differentiation during pregnancy through a program stimulated by steroid

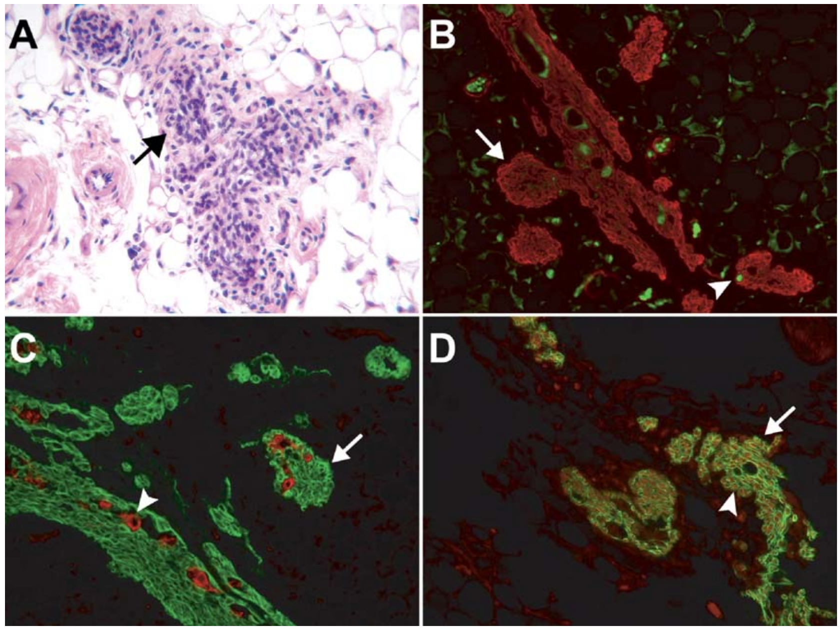

Fig. 10. Expansion of basal cells in *Pofut1*$^{fl/fl;MC}$ tissue during pregnancy. (A) H&E staining of a section from *Pofut1*$^{fl/fl;MC}$ mammary tissue at term. The epithelial compartment in *Pofut1*$^{fl/fl;MC}$ tissue consisted of densely packed cells with irregular dark nuclei. (B) Expression of SMA (red fluorescence) and Cre recombinase (green fluorescence). SMA staining demonstrated a massive increase in basal cells (arrow). Cre (arrowhead) was still found in some sporadic cells. (C) At term, the majority of cells (arrow) expressed K14 (green fluorescence). Cells expressing K18 (arrowhead) were sporadically interspersed in clusters of K14 positive cells. (D) Expression of p63 (red, arrowhead) and K5 (green, arrowhead).

and peptide hormones. The majority of proliferation occurs within the luminal compartment whereas proliferation is exceedingly rare in the basal (myoepithelial) compartment of the emerging alveolus (Anderson and Clarke, 2004; Joshi et al., 1986). This study demonstrates that the Notch pathway signaling through the transcription factor RBP-J is critical for the expansion and maintenance of alveolar luminal cells during pregnancy and the suppression of basal cell proliferation (for a summary see Fig. 11). In the absence of RBP-J, luminal cells acquire basal characteristics during pregnancy and acute

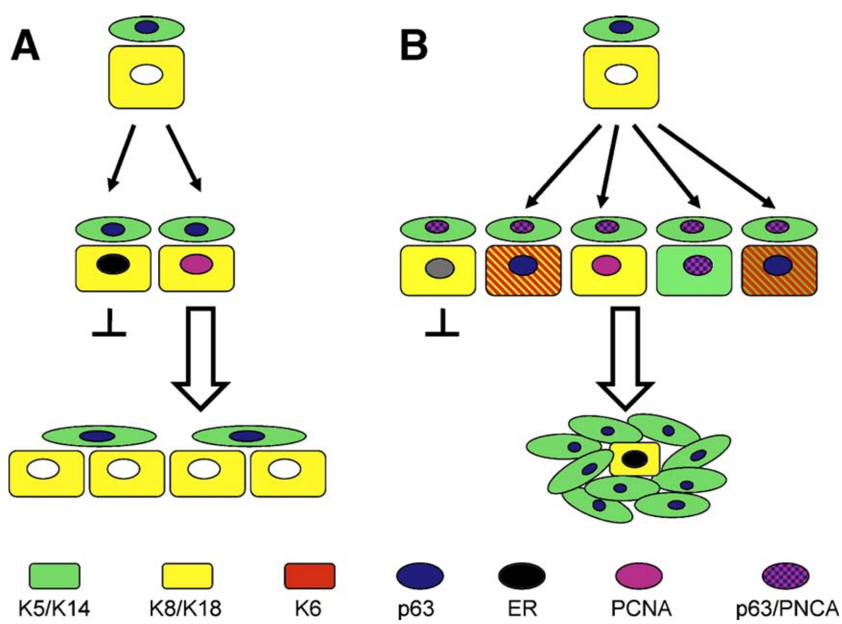

Fig. 11. Proposed model for the role of RBP-J in mammary development during pregnancy. (A) Upon receiving a pregnancy hormone stimulus, luminal cells (yellow) that do not express the ERα (black) undergo proliferation (indicated by the presence of PCNA in pink) and differentiate to form functional alveoli. Basal (myoepithelial) cells express basal cytokeratins (green) and p63 (blue) in their nuclei. (B) In the absence of RBP-J, some cells in the luminal compartment express p63 (blue). Aberrant expression of K6 (red) and basal cytokeratins (green) are transiently seen in luminal cells. Transiently, cells that coexpress K6 and K5/K14 and cells coexpressing K6 and K8 are seen. High levels of proliferation in the basal compartment lead to the accumulation of cells expressing basal markers (green) and p63. At the end of pregnancy, only a small number of genuine luminal cells (yellow), which also expressed the ERα (black), were present.

hormonal stimulation, and engage in extensive proliferation, which results in a disorganized assembly of myoepithelial-like cells. These cells prominently display expression of p63, K5/K14, and SMA, thereby establishing them as *bona-fide* basal or myoepithelial cells. Similarly, in the absence of Pofut1, which also results in inactive Notch receptors by a different mechanism, mammary epithelium acquires a basal character during pregnancy. This result is in agreement with previous experiments carried out in mice and *Drosophila*, which demonstrated that the deletion or down-regulation of Pofut1 results in phenotypes resembling those observed after loss of downstream effectors of the Notch signaling pathway (Okajima and Irvine, 2002; Shi and Stanley, 2003). Thus, Pofut1 appears as an important regulator of the Notch signaling pathway during mouse mammary gland development. We propose that the canonical Notch/RBP-J pathway is required for the maintenance of luminal cells and permits their preferential proliferation during pregnancy through the suppression of p63, which itself is required for the establishment of basal cells.

A recent study using primary human mammary cells in a 3D in vitro culture system has concluded that Notch signaling has a dual function during mammary development in that it promotes self-renewal of stem cells and progenitor cells and controls lineage-specific differentiation (Dontu et al., 2004). These authors demonstrated that the activation of Notch signaling in vitro led to increased formation of mammospheres and a more than 10-fold increase of myoepithelial progenitors. Conclusions from these gain-of-function in vitro studies are different from our in vivo loss-of-function studies. It is possible that the aberrant ectopic activation of Notch signaling in vitro induces RBP-J-independent pathways. Alternatively, there might be fundamental differences in mammary progenitor cells between mouse and human.

In the absence of RBP-J, ducts and immature alveoli in virgin mice appear to be normal with luminal cells expressing K8 and a subset expressing the ERα, and basal cells expressing K5 and p63. However, upon induction of alveolar differentiation, either through hormone injection or pregnancy, these cells altered their fate. An early event upon hormone stimulation was the accumulation of p63 in the luminal compartment and a massive proliferation of p63-positive cells. In the mouse, the presence of the basal cell transcription factor p63 is absolutely required for the establishment of epithelial structures, including skin and mammary glands (Mills et al., 1999; Yang et al., 1999). High levels of p63 are found in basal or proliferating cells of many tissues, in particular stem and progenitor cells (Pellegrini et al., 2001). In the epidermis, p63 has a dual role in that it acts as a molecular switch directing the commitment of basal cells, and its presence maintains an immature state characterized by extensive proliferation (Koster et al., 2004). Notably, p63 by itself can induce the expression of the basal cell marker K5 in a variety of unrelated cell lines as well as in mice (Koster et al., 2004). Overexpression of the p63 TA isoform in the simple epithelium lining the bronchioles in the lung resulted in their excessive proliferation and impaired terminal differentiation. Loss of RBP-J in mammary epithelium resulted in the accumulation of p63 in luminal cells, which we propose triggered two events. p63-positive luminal cells lost their luminal character and acquired a basal character as evidenced by the expression of K5. Moreover, p63-positive cells proliferated extensively.

Upon loss of RBP-J from mammary epithelium, a transient expansion of K6-positive luminal cells occurred. Bipotent progenitor cells, which give rise to luminal and basal cells, are characterized by the presence of K6 (Smith et al., 1990). While K6 is present in a few body cells in rapidly proliferating terminal end buds, it is rarely detected in developing ducts and alveoli during puberty and pregnancy. This suggests that, upon loss of RBP-J, luminal cells are initially arrested in an immature stage and fail to progress in their differentiation. An increased population of K6-positive cells was also observed in C/EBPβ-null mammary tissue (Grimm et al., 2002). These authors speculated that mutant cells could not progress past a progenitor stage. However, an increase of K6-positive cells is not a non-specific response to the loss of genes controlling mammary epithelial cell proliferation and differentiation, as PrlR- and Stat5-null mammary tissues do not display elevated numbers of K6 expressing cells (Grimm et al., 2002). The increase of K6-positive cells in transgenic mice expressing oncogenes activating the wnt pathway was interpreted as an enrichment of progenitor cells that constituted the basis for the development of mammary tumors (Li et al., 2003b). K6 has also been linked to wound healing and hyperproliferative disease (Wojcik et al., 2000). In the case of normal mammary tissue, the function of K6 is not clear. No K6 expression is found in wild-type luminal cells that undergo extensive proliferation during pregnancy. Moreover, there is no evidence that the K6-positive cells in RBP-J-null luminal cells undergo extensive proliferation.

It has been proposed that information exchanged between basal and luminal cells is critical for the controlled development of mammary epithelia (for review see Deugnier et al., 2002). Notably, ectopic expression of genes in the basal compartment can modulate the differentiation and growth of luminal cells (Teuliere et al., 2005). Through the inactivation of RBP-J in mammary progenitor cells, Notch signaling in developing alveoli was disrupted, which resulted in luminal cells losing their characteristics and acquiring salient features of basal cells. Studies with purified primary mammary cells demonstrated that the interaction between basal and luminal cells is essential for the maintenance of luminal cells (Pechoux et al., 1999). Purified luminal cells have the programmatic capacity to adapt a basal cell fate, but basal cells cannot convert to luminal cells. In this case, luminal cells that did not receive signals from the basal compartment lost their luminal characteristics. We propose that the fate of a luminal cell is unstable and influenced by its environment. In the absence of correct cues, luminal cell can adopt a basal cell fate as shown in the absence of RBP-J. Moreover, the ectopic activation of β-catenin (Miyoshi et al., 2002) or the loss of Smad4 (Li et al., 2003a) also results in transdifferentiation of luminal cells. In that case, expression of K5 is a transient state and the cells continue to differentiate into keratinocytes.

The function of approximately 100 proteins in mammary development and function has been studied in mice by gene deletion, either in the germline or specifically in mammary epithelium. While many of these proteins control cell proliferation and differentiation, to our knowledge, the *Rupsh* gene is the first one to shed light on the molecular mechanisms employed by the progenitor cells to ensure the controlled maintenance and proliferation of basal and luminal cells. We propose that Notch signaling through RBP-J suppresses p63 in luminal cells and therefore controls their maintenance and proliferation during pregnancy. Moreover, in the absence of Notch signaling, basal cells escape the proliferative block normally in place. The inactivation of RBP-J in other cell types, employing the mice used also in this study, has provided additional information on the common and cell-specific contributions of Notch signaling. Deletion of RBP-J from follicular stem cells results in an aberrant cell fate switch, which leads to the establishment of epidermal progenitors and basal cells expressing K5 (Yamamoto et al., 2003). Normal differentiation into hair cells was not seen in the absence of RBP-J, rather accelerated differentiation into epidermis. Thus both in mammary epithelium and in follicular cells, RBP-J is required for the maintenance of highly specialized cell types, luminal cells, and follicles. In the intestine, loss of RBP-J leads to a conversion of proliferating crypt cells into post-mitotic goblet cells (van Es et al., 2005). The prostate gland shares a number of features with mammary tissue, including the existence of two distinct cell layers within the epithelium, a luminal and a basal cell layer. Inactivation of Notch1 signaling in prostate epithelium leads to disrupted tissue architecture containing cells that express both luminal and basal markers (Wang et al., 2006), further supporting the notion that Notch signaling is required for the controlled expansion of lineages in mammary and prostate tissue, organs that are under hormonal control.

Acknowledgments

This research was supported by the Intramural Research Program of the NIH, NIDDK and NCI grant RO1 30645 to PS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2006.02.043.

References

Anderson, E., Clarke, R.B., 2004. Steroid receptors and cell cycle in normal mammary epithelium. J. Mammary Gland Biol. Neoplasia 9, 3–13.

Beres, T.M., Masui, T., Swift, G.H., Shi, L., Henke, R.M., Macdonald, R.J., 2006. PTF1 is an organ-specific and notch-independent basic helix–loop–helix complex containing the mammalian suppressor of hairless (RBP-J) or its paralogue, RBP-L. Mol. Cell. Biol. 26, 117–130.

Cui, Y., Riedlinger, G., Miyoshi, K., Tang, W., Li, C., Deng, C.X., Robinson, G.W., Hennighausen, L., 2004. Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. Mol. Cell. Biol. 24, 8037–8047.

DeOme, K.B., Faulkin Jr., L.J., Bern, H.A., Blair, P.B., 1959. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res. 19, 515–520.

Deugnier, M.A., Teuliere, J., Faraldo, M.M., Thiery, J.P., Glukhova, M.A., 2002. The importance of being a myoepithelial cell. Breast Cancer Res. 4, 224–230.

Dontu, G., Jackson, K.W., McNicholas, E., Kawamura, M.J., Abdallah, W.M., Wicha, M.S., 2004. Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. Breast Cancer Res. 6, R605–R615.

Gallahan, D., Callahan, R., 1987. Mammary tumorigenesis in feral mice: identification of a new int locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. J. Virol. 61, 66–74.

Gallahan, D., Callahan, R., 1997. The mouse mammary tumor associated gene INT3 is a unique member of the NOTCH gene family (NOTCH4). Oncogene 14, 1883–1890.

Gallahan, D., Jhappan, C., Robinson, G., Hennighausen, L., Sharp, R., Kordon, E., Callahan, R., Merlino, G., Smith, G.H., 1996. Expression of a truncated Int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis. Cancer Res. 56, 1775–1785.

Grimm, S.L., Seagroves, T.N., Kabotyanski, E.B., Hovey, R.C., Vonderhaar, B.K., Lydon, J.P., Miyoshi, K., Hennighausen, L., Ormandy, C.J., Lee, A.V., Stull, M.A., Wood, T.L., Rosen, J.M., 2002. Disruption of steroid and prolactin receptor patterning in the mammary gland correlates with a block in lobuloalveolar development. Mol. Endocrinol. 16, 2675–2691.

Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K., Honjo, T., 2002. Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. Int. Immunol. 14, 637–645.

Hennighausen, L., Robinson, G.W., 2001. Signaling pathways in mammary gland development. Dev. Cell. 1, 467–475.

Hennighausen, L., Robinson, G.W., 2005. Information networks in the mammary gland. Nat. Rev., Mol. Cell Biol. 6, 715–725.

Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G.H., Merlino, G., Callahan, R., 1992. Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. Genes Dev. 6, 345–355.

Joshi, K., Smith, J.A., Perusinghe, N., Monoghan, P., 1986. Cell proliferation in the human mammary epithelium. Differential contribution by epithelial and myoepithelial cells. Am. J. Pathol. 124, 199–206.

Kiaris, H., Politi, K., Grimm, L.M., Szabolcs, M., Fisher, P., Efstratiadis, A., Artavanis-Tsakonas, S., 2004. Modulation of notch signaling elicits signature tumors and inhibits hras1-induced oncogenesis in the mouse mammary epithelium. Am. J. Pathol. 165, 695–705.

Kordon, E.C., Smith, G.H., 1998. An entire functional mammary gland may comprise the progeny from a single cell. Development 125, 1921–1930.

Kordon, E.C., Smith, G.H., Callahan, R., Gallahan, D., 1995. A novel non-mouse mammary tumor virus activation of the Int-3 gene in a spontaneous mouse mammary tumor. J. Virol. 69, 8066–8069.

Koster, M.I., Kim, S., Mills, A.A., DeMago, F.J., Roop, D.R., 2004. p63 is the molecular switch for initiation of an epithelial stratification program. Genes Dev. 18, 126–131.

Krebs, L.T., Xue, Y., Norton, C.R., Shutter, J.R., Maguire, M., Sundberg, J.P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., Smith, G.H., Stark, K.L., Gridley, T., 2000. Notch signaling is essential for vascular morphogenesis in mice. Genes Dev. 14, 1343–1352.

Li, W., Qiao, W., Chen, L., Xu, X., Yang, X., Li, D., Li, C., Brodie, S.G., Meguid, M.M., Hennighausen, L., Deng, C.X., 2003a. Squamous cell carcinoma and mammary abscess formation through squamous metaplasia in Smad4/Dpc4 conditional knockout mice. Development 130, 6143–6153.

Li, Y., Welm, B., Podsypanina, K., Huang, S., Chamorro, M., Zhang, X., Rowlands, T., Egeblad, M., Cowin, P., Werb, Z., Tan, L.K., Rosen, J.M., Varmus, H.E., 2003b. Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proc. Natl. Acad. Sci. U. S. A. 100, 15853–15858.

Liu, X., Robinson, G.W., Hennighausen, L., 1996. Activation of Stat5a and Stat5b by tyrosine phosphorylation is tightly linked to mammary gland differentiation. Mol. Endocrinol. 10, 1496–1506.

MacKenzie, F., Duriez, P., Wong, F., Noseda, M., Karsan, A., 2004. Notch4 inhibits endothelial apoptosis via RBP-Jkappa-dependent and -independent pathways. J. Biol. Chem. 279, 11657–11663.

McKeon, F., 2004. p63 and the epithelial stem cell: more than status quo? Genes Dev. 18, 465–469.

Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R., Bradley, A., 1999. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398, 708–713.

Miyoshi, K., Shillingford, J.M., Smith, G.H., Grimm, S.L., Wagner, K.U., Oka, T., Rosen, J.M., Robinson, G.W., Hennighausen, L., 2001. Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. J. Cell Biol. 155, 531–542.

Miyoshi, K., Shillingford, J.M., Le Provost, F., Gounari, F., Bronson, R., von Boehmer, H., Taketo, M.M., Cardiff, R.D., Hennighausen, L., Khazaie, K., 2002. Activation of beta-catenin signaling in differentiated mammary secretory cells induces transdifferentiation into epidermis and squamous metaplasias. Proc. Natl. Acad. Sci. U. S. A. 99, 219–224.

Nielsen, T.O., Hsu, F.D., Jensen, K., Cheang, M., Karaca, G., Hu, Z., Hernandez-Boussard, T., Livasy, C., Cowan, D., Dressler, L., Akslen, L.A., Ragaz, J., Gown, A.M., Gilks, C.B., van de Rijn, M., Perou, C.M., 2004. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin. Cancer Res. 10, 5367–5374.

Oka, C., Nakano, T., Wakeham, A., de la Pompa, J.L., Mori, C., Sakai, T., Okazaki, S., Kawaichi, M., Shiota, K., Mak, T.W., Honjo, T., 1995. Disruption of the mouse RBP-J kappa gene results in early embryonic death. Development 121, 3291–3301.

Okajima, T., Irvine, K.D., 2002. Regulation of notch signaling by o-linked fucose. Cell 111, 893–904.

Ormandy, C.J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Edery, M., Brousse, N., Babinet, C., Binart, N., Kelly, P.A., 1997. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev. 11, 167–178.

Pechoux, C., Gudjonsson, T., Ronnov-Jessen, L., Bissell, M.J., Petersen, O.W., 1999. Human mammary luminal epithelial cells contain progenitors to myoepithelial cells. Dev. Biol. 206, 88–99.

Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., Ponzin, D., McKeon, F., De Luca, M., 2001. p63 identifies keratinocyte stem cells. Proc. Natl. Acad. Sci. U. S. A. 98, 3156–3161.

Raafat, A., Bargo, S., Anver, M.R., Callahan, R., 2004. Mammary development and tumorigenesis in mice expressing a truncated human Notch4/Int3 intracellular domain (h-Int3sh). Oncogene 23, 9401–9407.

Robinson, G.W., Wagner, K.U., Hennighausen, L., 2001. Functional mammary gland development and oncogene-induced tumor formation are not affected by the absence of the retinoblastoma gene. Oncogene 20, 7115–7119.

Sapino, A., Macri, L., Gugliotta, P., Bussolati, G., 1990. Immunocytochemical identification of proliferating cell types in mouse mammary gland. J. Histochem. Cytochem. 38, 1541–1547.

Sasamura, T., Sasaki, N., Miyashita, F., Nakao, S., Ishikawa, H.O., Ito, M., Kitagawa, M., Harigaya, K., Spana, E., Bilder, D., Perrimon, N., Matsuno, K., 2003. Neurotic, a novel maternal neurogenic gene, encodes an *O*-fucosyltransferase that is essential for Notch–Delta interactions. Development 130, 4785–4795.

Schweisguth, F., 2004. Notch signaling activity. Curr. Biol. 14, R129–R138.

Shi, S., Stanley, P., 2003. Protein *O*-fucosyltransferase 1 is an essential component of Notch signaling pathways. Proc. Natl. Acad. Sci. U. S. A. 100, 5234–5239.

Shi, S., Stahl, M., Lu, L., Stanley, P., 2005. Canonical Notch signaling is dispensable for early cell fate specifications in mammals. Mol. Cell. Biol. 25, 9503–9508.

Shillingford, J.M., Miyoshi, K., Robinson, G.W., Grimm, S.L., Rosen, J.M., Neubauer, H., Pfeffer, K., Hennighausen, L., 2002. Jak2 is an essential tyrosine kinase involved in pregnancy-mediated development of mammary secretory epithelium. Mol. Endocrinol. 16, 563–570.

Shyamala, G., Chou, Y.C., Louie, S.G., Guzman, R.C., Smith, G.H., Nandi, S., 2002. Cellular expression of estrogen and progesterone receptors in mammary glands: regulation by hormones, development and aging. J. Steroid Biochem. Mol. Biol. 80, 137–148.

Smith, G.H., Boulanger, C.A., 2003. Mammary epithelial stem cells: transplantation and self-renewal analysis. Cell Proliferation 36 (Suppl. 1), 3–15.

Smith, G.H., Mehrel, T., Roop, D.R., 1990. Differential keratin gene expression in developing, differentiating, preneoplastic, and neoplastic mouse mammary epithelium. Cell Growth Differ. 1, 161–170.

Tanigaki, K., Kuroda, K., Han, H., Honjo, T., 2003. Regulation of B cell development by Notch/RBP-J signaling. Semin. Immunol. 15, 113–119.

Teuliere, J., Faraldo, M.M., Deugnier, M.A., Shtutman, M., Ben-Ze'ev, A., Thiery, J.P., Glukhova, M.A., 2005. Targeted activation of beta-catenin signaling in basal mammary epithelial cells affects mammary development and leads to hyperplasia. Development 132, 267–277.

van Es, J.H., van Gijn, M.E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D.J., Radtke, F., Clevers, H., 2005. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 435, 959–963.

Wagner, K.U., Wall, R.J., St-Onge, L., Gruss, P., Wynshaw-Boris, A., Garrett, L., Li, M., Furth, P.A., Hennighausen, L., 1997. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res. 25, 4323–4330.

Wagner, K.U., McAllister, K., Ward, T., Davis, B., Wiseman, R., Hennighausen, L., 2001. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res. 10, 545–553.

Wang, X.D., Leow, C.C., Zha, J., Tang, Z., Modrusan, Z., Radtke, F., Aguet, M., de Sauvage, F.J., Gao, W.Q., 2006. Notch signaling is required for normal prostatic epithelial cell proliferation and differentiation. Dev. Biol. 290, 66–80.

Wojcik, S.M., Bundman, D.S., Roop, D.R., 2000. Delayed wound healing in keratin 6a knockout mice. Mol. Cell. Biol. 20, 5248–5255.

Yamamoto, N., Tanigaki, K., Han, H., Hiai, H., Honjo, T., 2003. Notch/RBP-J signaling regulates epidermis/hair fate determination of hair follicular stem cells. Curr. Biol. 13, 333–338.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R.T., Tabin, C., Sharpe, A., Caput, D., Crum, C., McKeon, F., 1999. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398, 714–718.